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Statistical Criteria to Establish Optimal Antibody Dilution in Flow Cytometry Analysis

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Background: In direct techniques of flow cytometry, the optimal antibody dilution or titer point is established from the plateau area of the antibody titration curve. However, the plateau area is defined without any statistical criteria, which may lead to an incorrect selection of antibody dilution. Herein, we report statistical criteria to establish the optimal antibody dilution for CD14, CD8, CD4, and CD3 analysis by flow cytometry in peripheral whole blood.

Methods: The unpaired *t*-test (two-tail *P* value) was used as statistical criteria to analyze the titration curve of the following monoclonal antibody panels: CD14-FITC, CD8-FITC, CD4-RD1, and CD3-PC5.

Results: Using the unpaired *t*-test (two-tail *P* value), the plateau area from the antibody titration curve was fitted when two consecutive antibody volumes showed mean peak of channel fluorescence (MPCF) values not significantly different. When the antibody was used at volume corresponding to that of the antibody titration point, the flow cytometry analysis of whole blood samples with different density of cell antigens can be correctly discriminated.

Conclusion: This statistical criteria allows the fitting of the plateau area of MPCF versus antibody volume and consequently, to define the optimal antibody dilution. © 2007 Clinical Cytometry Society

Key terms: statistical criteria; unpaired *t*-test; antibody titration; flow cytometry; mean peak of channel fluorescence (MPCF)

The importance of performing flow cytometry analysis in research and clinical practice is well established. Usually, the value of the mean peak of channel fluorescence (MPCF) is considered to be directly proportional to the amount of antigen expressed in a determined cell type. For this condition to be valid, among other aspects of the assay, the proper use of the antibodies is essential. The determination of the antibody dilution constitutes the key step previous to flow cytometry analysis, since it is highly dependent on the density of antigen in cells. Ideally, every antibody concentration should be established for each sample that requires analysis. However, this is not feasible, both in terms of time and resources. Usually, normal and pathologic samples, which are known to express the relevant antigen, are used to obtain the optimal antibody dilution. When direct techniques of flow cytometry are applied to whole blood, different volumes of directly labeled antibody are added to a fixed volume of whole blood, giving rise to doubling dilutions of antibody (1,2). Then, the mixtures are analyzed by flow cytometry and the results are plotted on two types of graphs: (i) MPCF against antibody dilution (linear/log scale) and (ii) percentage of cell positivity against antibody dilution (linear/log scale). The optimal antibody dilution or titration point is the one that lies on the plateau area of both plots, i.e. a dilution that results in maximal fluorescence and cell positivity (1,2). However, in this procedure the plateau area is defined without any statistical criteria, which may lead to an incorrect selection of the optimal antibody dilution. In this

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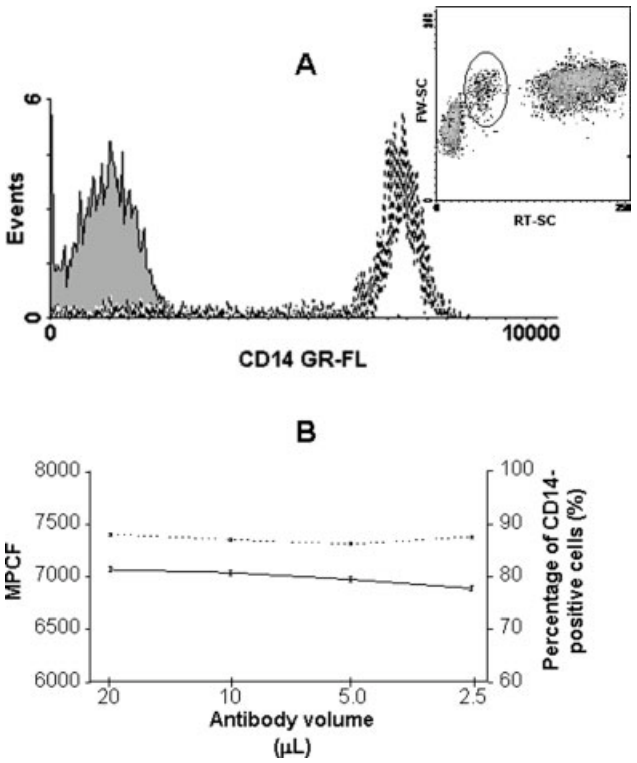

FIG. 1. **A**: CD14 histogram in monocytes from peripheral whole blood with normal density of CD14 antigen, using variable volumes of the monoclonal FITC-conjugated anti-CD14 antibody. Insert: Dot-plot FW-SC versus RT-SC graph indicating the monocyte gate (circle). **B**: The antibody titration curve in monocytes with normal CD14 antigen density indicating the MPCF (left-y scale, solid line) and percentage of CD14-positive cells (right-y scale, dotted line) versus antibody volumes.

sense, using an incorrect antibody dilution would produce a significant bias in the antigen measurement and consequently, an erroneous interpretation of a determined biological or clinical process.

In our laboratory, we normally use statistical criteria to define the optimal antibody dilution from the plateau area of MPCF versus antibody dilution. In this work, we report statistical criteria to establish the optimal antibody dilution for the flow cytometry analysis of CD14 in monocytes and CD8, CD4, and CD3 in lymphocytes from peripheral whole blood. From the results obtained, we proposed that these statistical criteria can be extrapolated to other situations that require flow cytometry analysis using direct conjugated antibodies to measure specific antigens in cells.

For this study, different whole blood samples were used for the titration of the antibodies raised CD14-monocyte and CD8/CD4/CD3-lymphocyte antigens. In all cases, the flow cytometry analysis were performed in conformity with the ISO-15189:2005 accreditation program (http://oaa.org.ar/200504/index.asp) and CDC guidelines (3). Each monoclonal antibody was directly labeled with a different dye: fluorescein isothiocyanate (FITC), R-phycoerythrin (RD1), and phycoerythrin-Cy5 (PC5). The panels of monoclonal antibodies used were: CD8-FITC/CD4-RD1/CD3-PC5 (Beckman Coulter, Ireland, Clones SFCI21ThyD3, SFCI12T4D11, and UCHT1, respectively), and CD14-FITC (Immunotech, France, Clone RMO52), which were used following the manufacturer's protocols. At least 5,000 gated lymphocytes and 1,000 gated monocytes were acquired in each sample using the flow cytometer Cytoron Absolute (Ortho Diagnostic System, Raritan, NJ), operated with Immunocount II software. The data was analyzed by WinMDI 2.8 software (J. Trotter, Scripps Research Institute, La Jolla, CA). In this way, we obtained the MPCF values, coefficient of variation (CV%), number of lymphocytes and monocytes present in the gate (by dot-plot FW-SC versus RT-SC), and percentage of cell expressing CD3 (CD3+CD4+ and CD3+CD8+) in the lymphocyte gate, and CD14 in the monocyte gate (by dot-plot CD3+ and CD14+ versus RT-SC). The statistical criteria used to compare different volumes of antibody was the unpaired *t*-test (two-tail *P* value) using GraphPad Instat 3.06 for Windows 95 (GraphPad Software, San Diego CA, www.graphpad.com).

Table 1
Unpaired t-*Test to Compare MPCF Values Obtained from Different Antibody Dilutions*

Antibodies	Comparison (μL)	Mean difference (MPCF)	*t*	*P*-value
CD14	20 vs. 10	−28	1.817 (NS)	>0.05*
	10 vs. 5.0	−61	4.002 (***)	<0.0001*
	5.0 vs. 2.5	−83	5.470 (***)	<0.0001**
CD8	20 vs. 15	−6	0.482 (NS)	>0.05**
	15 vs. 10	−55	4.330 (***)	<0.0001**
	10 vs. 5.0	−57	4.688 (***)	<0.0001**
CD4	20 vs. 15	−4	0.662 (NS)	>0.05**
	15 vs. 10	−40	6.437 (***)	<0.0001**
	10 vs. 5.0	−25	4.081 (***)	<0.0001**
CD3	20 vs. 15	−2	0.227 (NS)	>0.05**
	15 vs. 10	−38	4.125 (***)	<0.0001**
	10 vs. 5.0	−57	6.133 (***)	<0.0001**

NS, not significant.
*Without Welch correction. The *F* test suggests that the difference between the two standard deviations is not significant.
**With Welch correction. The *F* test suggests that the difference between the two standard deviations is significant.
***If the value of *t* is greater than 1.960 then the *P*-value is less than 0.05 and differences are significant.

When the variance parameter was not homogeneous, we used the Welch correction in the unpaired *t*-test. The protocol of antibody titration for CD14 analysis was as follows: 20, 10, 5, and 2.5 μL of monoclonal FITC-conjugated anti-CD14 antibody were added to 50 μL aliquots of the same whole blood sample, containing normal or high density of CD14-monocyte antigen. Considering the manufacturer's recommendation of 20 μL of antibody per 5 $\times$ 10^5 target cells, in this study the monocyte number in 50 μL aliquots of whole blood ranged from 3.0 $\times$ 10^4 to 5.0 $\times$ 10^4 cells. Hence, in the range of antibody dilution assayed, the proportion of antibody volume per monocyte number exceeded the proportion of antibody volume per target cells recommended by the manufacturer. The protocol of antibody titration for CD8, CD4, and CD3 analysis was as follows: 20, 15, 10, and 5 μL of a three-color fluorescent reagent comprising the three monoclonal antibodies were added to 50 μL aliquots of the same whole blood sample. Following manufacturer's recommendations, three different whole blood samples with leukocyte values ranging from 3.0 $\times$ 10^3/μL to 10.0 $\times$ 10^3/μL were used for titration of each antibody, being the antibody volume/whole blood volume ratio at least $\geq$0.1

Figure 1A shows the CD14 histograms from the monocyte gate of peripheral whole blood analysis with normal density of CD14 antigen, using variable volumes of the monoclonal FITC-conjugated anti-CD14 antibody. Figure 1B shows the titering curve of antibody dilution in relation to the MPCF and percentage of CD14-positive cells. Following nonstatistical criteria (1), the optimal antibody dilution could be established from 2.5 μL up to 20 μL without apparent modification of the MPCF or the percentage of CD14-positive cells. Hence, this dilution range of antibody might be considered to have reached the plateau area of this antibody titration. In the same way, it could be established that each volume of antibody tested is representing a similar titer point for this antibody. However, by using the statistical criteria of the unpaired *t*-test (two-tail *P* value), we demonstrated that between 10 and 20 μL of antibody volume the values of MPCF were not significantly different, while between 5 and 10 μL as well as between 2.5 and 5 μL of antibody volume the values of MPCF were significantly dissimilar (Table 1). Hence, considering this statistical criteria it can be deduced that the plateau area in MPCF is reached when using 10 to 20 μL of CD14 antibody.

We evaluated whole blood samples with high density of CD14 antigen per monocyte to compare both criteria to choose the optimal antibody dilution in relation to the amount of CD14 expressed on monocytes. Figure 2A shows the CD14 histograms from the monocyte gate of a representative individual containing high density of CD14 antigen, using variable volumes of the monoclonal FITC-conjugated anti-CD14 antibody. In Figure 2B (black bars), it is shown that antibody volumes of 2.5 and 5 μL yielded a significant negative bias ($P < 0.05$) in CD14 analysis respect to the normal reference values, whereas antibody volumes from 10 to 20 μL showed MPCF values of CD14 antigen higher than the upper limit of normal reference values. Similar results were obtained with other individuals containing high density of CD14 per monocytes (data not shown). When whole blood sample from a representative individual containing normal density of CD14 antigen in monocytes is used (Fig. 2B, white bars), we observed a significant negative bias respect to normal reference value only with 2.5 μL of antibody. In conclusion, these results indicate that the optimal titration point for the anti-CD14 antibody ranges from 10 to 20 μL, since whole blood samples with different density of cell antigens can be correctly discriminated.

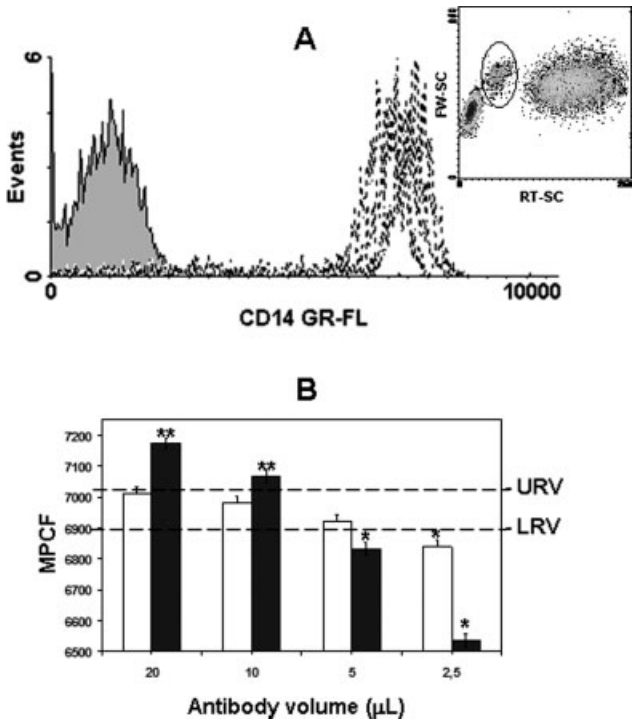

FIG. 2. **A**: CD14 histogram in monocytes from peripheral whole blood with high density of CD14 antigen, using variable volumes of the monoclonal FITC-conjugated anti-CD14 antibody. Insert: Dot-plot FW-SC versus RT-SC graph indicating the monocyte gate (circle). **B**: MPCF values obtained from monocytes of a representative individual containing high CD14 antigen (black bars) versus variable volumes of the monoclonal FITC-conjugated anti-CD14 antibody. As a control, it is also showed MPCF values from monocytes of a representative individual containing normal CD14 antigen (white bars) analyzed with different volumes of anti-CD14 antibody. The bars represent the mean ± 2 SEM of MPCF values obtained by triplicate experiments. The dotted lines indicate the confidence limits of 95% of normal reference MPCF values for CD14 in monocytes of normal individuals ($n = 19$) using 20 μL of anti-CD14 antibody. LRV, lower reference value; URV, upper reference value (URV). (*), significant negative bias ($P < 0.05$) respect to reference values; (**), significant difference ($P < 0.05$) respect to reference values.

In addition, this statistical criteria proposed to establish the optimal antibody dilution for flow cytometry was applied to the study of other cell antigens in lymphocytes of peripheral whole blood, such as CD8, CD4, and CD3. Following the manufacturer's recommendations (of antibody volume/whole blood volume ratio $\geq$0.1), the antibody titration was performed using three different whole blood samples containing 3.0 $\times$ 10^3,

6.2×10^3, and 10.0×10^3 leukocytes/μL. Statistical analysis obtained with whole blood containing 6.2×10^3 leukocytes/μL and different antibody volumes of anti-CD8, anti-CD4, and anti-CD3 antibodies is shown in Table 1. In this case, we have demonstrated that between 15 and 20 μL of antibody, the values of MPCF were not significantly different for each antibody analyzed, while between 10 and 15 μL as well as 5 and 10 μL of antibody volume, the values of MPCF were significantly dissimilar. Similar results of statistical significance were obtained when the antibody titration was performed with whole blood samples containing 3.0×10^3 and 10.0×10^3 leukocytes/μL (data not shown). Hence, considering this statistical criteria it can be deduced that the plateau area in MPCF is reached between 15 and 20 μL of the CD8, CD4, and CD3 antibodies. These statistical criteria was also applied in our laboratory for other cell antigens analyzed by flow cytometry, including CD19 and CD56 in lymphocytes of peripheral whole blood (data not shown).

In conclusion, we propose a statistical criteria in order to establish the optimal antibody dilution for flow cytometry with a better analytical confidence, providing a reliable tool to measure antigens expressed on different cell types. This statistical criteria allows the fitting of the plateau area of MPCF versus antibody volume and consequently, to define the optimal antibody dilution. A high antigen density in cells can modify the accuracy of the flow cytometry analysis, basically influenced by the level of saturation of the antigen–antibody reaction. Statistical criteria are currently used in different analytical procedures as well as in method validation. In this sense, the use of statistical criteria for the selection of the optimal antibody dilution might in future be considered indispensable for quality management in flow cytometry, constituting a reliable analytical procedure in terms of accuracy and reproducibility of antigen measurement in different cell types.

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